Engineering bone tissue from human embryonic stem cells

Darja Marolt1, Iván Marcos Campos4, Sarindr Bhumiratana4, Ana Koren1,2, Petros Petridis1, Geping Zhang5, Patrice F. Spitalnik6, Warren L. Grayson6, and Gordana Vunjak-Novakovic1,2

1Department of Biomedical Engineering, and 2Department of Pathology and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, NY 10032; 3Blood Transfusion Centre of Slovenia, Ljubljana, SI-1000, Slovenia; and 4Department of Biomedical Engineering, School of Medicine, The Johns Hopkins University, Baltimore, MD 21231

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In extensive bone defects, tissue damage and hypoxia lead to cell death, resulting in slow and incomplete healing. Human embryonic stem cells (hESC) can give rise to all specialized lineages found in healthy bone and are therefore uniquely suited to aid regeneration of damaged bone. We show that the cultivation of hESC-derived mesenchymal progenitors on 3D osteoconductive scaffolds in bioreactors with medium perfusion leads to the formation of large and compact bone constructs. Notably, the implantation of engineered bone in immunodeficient mice for 8 wk resulted in the maintenance and maturation of bone matrix, without the formation of teratomas that is consistently observed when undifferentiated hESCs are implanted, alone or in bone scaffolds. Our study provides a proof of principle that tissue-engineering protocols can be successfully applied to hESC progenitors to grow bone grafts for use in basic and translational studies.

Results and Discussion
Bone Tissue Engineering Model System. We developed a stepwise protocol to engineer bone-like constructs from hESC (Fig. 1). To mimic the progression of bone development, which starts with condensation of mesenchymal cells (29), we induced mesenchymal differentiation of hESC in serum-supplemented medium. Subsequently, hESC-derived progenitors were differentiated into bone by cultivation in osteoconductive scaffolds using perfusion bioreactors with interstitial flow of osteoinductive culture medium (30, 31). Because hESC can differentiate into mixed populations of cells that pose a risk of subsequent tumor formation (17), the phenotypic stability of engineered bone was evaluated in SCID-beige mice.

Derivation of Mesenchymal Progenitors of hESCs. Undifferentiated hESCs give rise to cells resembling the mesenchymal stem cells either spontaneously, in coculture with adult cells, or after exposure to differentiation-inducing factors (12–15). We induced the differentiation of two lines of hESC (H9 and H13) in monolayer culture by simply supplementing culture medium with


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1Present address: The New York Stem Cell Foundation, New York, NY 10032.
2To whom correspondence should be addressed. E-mail: g2131@columbia.edu.

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serum and eliminating bFGF for 7 d, after which the cultures were passaged. Between passages 1 and 3, our protocol resulted in adherent progenitors exhibiting continuous growth (Fig. S1A), fibroblastic morphology (Fig. S1A), and homogenous expression of mesenchymal surface antigens CD44, CD73, CD90, CD105, and CD166 (Fig. S1C). Antigens marking pluripotent stem cells (SSEA-4), early differentiation (SSEA-1), endothelial- (CD31), hematopoietic- (CD34), and neuroectodermal-mesenchymal (CD271) lineages were not expressed (Fig. S1C).

hESC-derived progenitors exhibited the potential for mesenchymal differentiation in vitro comparable to that of BMSC (Fig. S1B) (32). Strong osteogenic potential of hESC progenitors was evidenced by alkaline phosphatase activity and matrix mineralization in osteogenic medium (Fig. S1B). Both the chondrogenic potential of cells cultured in chondrogenic medium that was evidenced by the presence of Alcian Blue-positive glycosaminoglycan and the adipogenic potential of cells cultured in adipogenic medium that was evidenced by the oil red staining were weaker for hESC progenitors than BMSCs (Fig. S1B). These findings are consistent with previous reports for other hESC lines (18, 33).

Robust Development of Bone Matrix in Perfused Bioreactor Cultures. We reported the formation of bone-like tissues by adult BMSCs cultured for 5 wk on decellularized bone scaffolds (30, 34, 35) and it has been shown that medium perfusion through cell-seeded scaffolds was critical for the viability and maturation of engineered bone (30, 34, 36–38). The flow velocities providing sufficient oxygen to maintain cell viability in large scaffolds ranged from 0.08 to 1.8 mm/s—with 0.8 mm/s being optimal for bone formation, corresponding to the initial fluid shear in the

Fig. 1. Bone engineering protocol and timeline. Undifferentiated hESC were cultured in mesoderm-inducing medium for 1 wk. Adherent cells were expanded in monolayer for 4 passages (3–4 wk) and seeded on decellularized bone scaffolds in osteogenic medium for 3 d to allow cell attachment. Cell-seeded constructs were then cultured in osteogenic medium for 5 wk in either perfusion bioreactors or static dishes. Tissue development was evaluated after 3 and 5 wk of culture. Bioreactor-grown bone was implanted s.c. in SCID-beige mice for 8 wk to evaluate tissue stability and maturation.

Fig. 2. Effects of bioreactor cultivation on bone tissue development. (A) DNA content per wet weight (ww) of tissue constructs was expressed as percent initial value (at the start of bioreactor/static cultivation) and found to be significantly higher in bioreactor groups (br) compared with the static group (st). (B) Similarly, significantly higher alkaline phosphatase (AP) activity was observed after 3 wk of culture in the H9 bioreactor group. Both DNA content and AP activity increased significantly in the static group (st) compared with the static group (st). (C) Cumulative osteopontin (OPN) release into culture medium was significantly higher after 2 wk of culture (medium change 4 and later) in the bioreactor groups than in static groups. Data represent averages ± SD (n = 3–5; P < 0.05; * and #, statistically significant differences from the H9 bioreactor and BMSC bioreactor groups at the same timepoint; $, a statistically significant difference within the group between week 3 and week 5). (D) Positive effects of bioreactor culture were corroborated by histological analyses, showing denser tissue deposition in the bioreactor groups compared with the static group after 5 wk of culture (Hematoxylin Eosin; Top). Deposition of bone matrix was confirmed by positive staining of collagen (Masson Trichrome, blue color; Middle Upper), osteocalcin (brown color; Middle Lower), and osteoid (Goldner Trichrome, red color; Bottom). Only minimal staining was observed in statically cultured constructs at weeks 3 and 5. Black T lines mark the position where low magnification micrographs were overlayed. Inset represents negative staining control.
range of 0.6 and 20 mPa, which increased with the deposition of a new bone matrix (30).

Bone tissue engineering protocols established in these studies for adult mesenchymal stem cells were applied in the present study to mesenchymal progenitors derived from hESCs, using the flow velocity of 0.8 mm/s. Our rationale was that the operating conditions shown optimal for BMSC (30) will also support the survival and formation of bone by hESC-derived mesenchymal progenitors. Perfused bioreactor cultures of hESC-progenitors had significantly higher cell numbers (Fig. 2A and Fig. S2A), alkaline phosphatase activity (Fig. 2B and Fig. S2B), osteopontin release (Fig. 2C and Fig. S2C), and bone matrix density (Fig. 2D and Fig. S2D) than the corresponding static cultures. The effects of perfusion were comparable for constructs grown by using hESC and BMSC (Fig. 2A–C). In comparison with the H9 line, the H13 line exhibited slightly lower cellularity and more variation in tissue density and distribution (Fig. 2 and Fig. S2), suggesting differences in attachment and/or growth pattern between different cell lines. Overall, the acceleration of tissue development in constructs engineered from both hESC and BMSC indicates the vital role of interstitial medium flow in bone formation (Fig. 2 and Fig. S2).

Histological examinations revealed that the matrix produced by hESC-derived progenitors and BMSC contained collagen, osteopontin, bone sialoprotein, and osteocalcin, which indicate the development and maturation of bone specific matrix (Fig. 2D and Fig. S3) and the formation of osteoids (Fig. 2D and Fig. S3B). In contrast to static cultures, which showed only scarce deposition of bone matrix, perfused cultures yielded dense bone matrix (Fig. 2 and Fig. S3), with the density of homogenously distributed bone matrix being comparable for perfused hESC-progenitor and BMSC constructs (Fig. S3). These data suggest that hESC-mesenchymal progenitors continue to mature toward bone-depositing osteoblasts.

The accumulation and maturation of bone-like tissue was confirmed by μCT imaging (Fig. 3 and Fig. S4) that evidenced significant increases in mineralized bone volume and trabecular thickness, along with significant decreases in trabecular spacing in perfused constructs formed from H9, H13, and BMSC (Fig. 3 and Fig. S4), confirming the progression of mineralization.

Our results demonstrate that tissue engineering parameters determined for BMSC can be translated to hESC-derived mesenchymal progenitors, with implication to engineering of clinical-size grafts (35), composite grafts (39), and testing of novel biomaterials (40). It remains to be determined whether hESC-mesenchymal progenitors derived by different protocols (12, 18) would exhibit similar functional properties.

Engineered Bone Tissue Remains Stable in Vivo, with Evidence of Maturation, Vascularization, and Remodeling. To establish safety of hESC-engineered bone constructs, it is critical to evaluate their phenotypic stability in an in vivo setting (17, 27). We selected to test the stability of engineered bone grafts in a s.c. transplantation model in immunodeficient mouse, which is frequently used to assess cells and scaffolds in bone tissue engineering, and for evaluation of pluripotent stem cells (17, 25).

Undifferentiated hESCs implanted either in Matrigel or in osteogenic scaffolds invariably formed teratomas containing lineages of all three germ layers by 7 wk in vivo (Fig. 4A). hESC-mesenchymal progenitors implanted on bone scaffolds after 3-d seeding but without further in vitro cultivation exhibited some signs of osteogenesis, shown by formation of loose connective tissue that was weakly positive for bone matrix proteins (Fig. 4A and B). The lack of evidence of other germ-layer tissues suggests that our induction protocol is sufficient to restrict the pluripotency of hESCs (confirmed by the presence of teratomas in hESC transplantation groups) to mesenchymal lineages. Similar absence of tumors has been reported in a recent study where hESC-mesenchymal progenitors were injected s.c. into immunodeficient mice for up to 6 mo (41). In another study, where bioreactor cultivation was not used to support in vitro bone maturation, mesenchymal induction resulted in teratomas between 8 and 20 wk in vivo, suggesting that longer studies may be needed to validate the safety of specific differentiation protocols (17).
alternative to the extended hESC-mesenchymal induction, cell purification by fluorescent-activated cell sorting for specific mesenchymal markers could be implemented (14).

Notably, engineered bone maintained dense homogenous bone protein matrix over 8 wk in vivo (Fig. 4B), with highest-density areas adjacent to the scaffold structure. Cell lineages that are not present in bone were not detected in these constructs. The human origin of the cells was confirmed by nuclear staining (Fig. S5). Bone explants were surrounded by loose connective tissue capsules and contained functional microvasculature containing blood cells in the interior of engineered tissues. We also observed osteoclast invasion at the construct edges, suggesting the initiation of scaffold resorption (Fig. S5).

The μCT examination of the engineered bone explanted after 8 wk in vivo supported continued maturation of engineered bone, with significant increases in bone volume, bone volume to tissue volume ratio, and trabecular thickness compared with the constructs at the time of implantation (after 5 wk of culture; Fig. 3). The total increases in bone volume—an average of 2.2 mm³ during the 5 wk of in vitro culture and an additional 3.4 mm³ during 8 wk in vivo—were within the ranges reported for new bone formation after implantation of growth-factor releasing scaffolds for repair of critically sized bone defects (42).

Together with the presence of osteoclasts invading the constructs in the periphery region, these findings suggest that the combined effects of osteoinductive bone scaffolds and bioreactor cultivation yield engineered constructs with potential for enhanced bone healing. The underlying mechanisms appear to involve active remodeling mediated by the implanted hESC-progenitors maturing into osteoblastic cells and the invading host cells (43).

In summary, we report that perfusion culture is critical for engineering centimeter-size bone grafts from hESC. The engineered bone tissue was stable for 8 wk in vivo and exhibited signs of continued bone development, indicating a potential for bone defect regeneration. Our study shows the possibility to translate the bone tissue engineering protocols developed for adult stem cells (such as those derived from bone marrow aspirates) to embryonic-like stem cells, thereby taking advantage of the potential of pluripotent cell sources for basic and translational studies. The lack of tissue types reminiscent of tumors in engineered bone constructs implanted for 8 wk in vivo suggests at least a short-term stability and safety of engineered bone grafts. Ongoing long-term studies in orthotopic implantation models will help evaluate the safety and functionality of bone grafts engineered from hESC derivatives.

Methods
Detailed experimental methods are provided as SI Methods.

Cell Culture. hESC (lines H9 and H13) were induced into mesenchymal lineage in serum-supplemented medium for 7 d, split, and subcultured for up to 55 d (10–11 passages). Expression of surface antigens was determined by flow cytometry. In vitro differentiation potential was evaluated in monolayers and pellet cultures, with BMSC serving as controls.

Decellularized Bone Scaffolds. Scaffolds were prepared as in our previous studies (30, 34).

Perfusion Bioreactor Culture. hESC-derived progenitors were seeded into scaffolds (1.5 million cells per scaffold) and cultured in osteogenic medium for 3 d to allow cell attachment, and then in either perfusion bioreactors or six-well plates for up to 5 wk (Fig. 1). Medium samples were taken for biochemical assays at each medium change (twice a week). Tissue constructs analyzed after 3 and 5 wk of cultivation (for details, see SI Methods).

Implantation. Safety and phenotype stability of perfused bone constructs from H9 progenitors were assessed over 8 wk of s.c. implantation in immunodeficient (SCID-beige) mice, according to the Columbia University Institutional Animal Care And Use Committee approved animal protocol. Controls consisted of (i) H9-mesenchymal progenitors seeded in scaffolds, (ii) undifferentiated H9 cells seeded in scaffolds, and (iii) undifferentiated H9 cells encapsulated in Matrigel.

Fig. 4. Stability of engineered bone implanted in vivo. Histological analysis indicated stability of mature bone phenotype in H9-engineered bone after 8 wk of s.c. implantation. (A) In contrast, a formation of loose connective tissue was detected in bone scaffolds seeded with H9-progenitors before implantation, and teratoma tissue was found in implants formed from undifferentiated H9 cells in either bone scaffolds or Matrigel. Brackets denote positions of high magnification images. (B) Quantitative histomorphometric analyses indicated significantly larger fractional area staining positively (brown color) for bone markers osteopontin (Top and Middle Top), bone sialoprotein (Middle) and osteocalcin (Middle Bottom), compared with scaffolds seeded with H9-derived progenitors. Insets represent negative staining controls. Similarly, significantly higher area fractions covered with osteoids (red color; Bottom) were detected in engineered bone compared with scaffolds seeded with H9-derived progenitors. Data represent average ± SD (n = 5; P < 0.01; *, statistically significant differences between the groups). Arrows mark the presence of microvessels. Black T lines mark the positions where low magnification micrographs were overlaid.


**Supporting Information**

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**SI Methods**

**Materials.** KnockOut DMEM (KO-DMEM), KnockOut serum replacement (KO-SR), basic fibroblast growth factor (bFGF), tumor growth factor β-3, 1-t-glutamine, β-mercaptoethanol, non-essential amino acids solution (100x), penicillin-streptomycin solution (100x), Insulin-Transferrin-Selenium supplement (ITS; 100x), and Dulbecco’s PBS (DPBS) were purchased from Invitrogen. Mouse embryonic fibroblasts were from Millipore. Porcine skin gelatin and tissue culture water were from Sigma-Aldrich. HyClone FBS, US origin was from Fisher Scientific. All other chemicals were purchased from Sigma-Aldrich unless otherwise noted.

**Differentiation and Characterization of Mesenchymal Progenitors from hESC.** hESC lines H9 and H13 were obtained from the WiCell Research Institute (Madison, WI) and expanded on mouse embryonic fibroblasts according to established protocols (WiCell Research Institute, Madison, WI; www.wicell.org). hESC expansion medium consisted of KO-DMEM supplemented with 20% (vol/vol) KO-SR, 5 ng/mL bFGF, 1 mM t-glutamine, 0.1 mM β-mercaptoethanol, and 0.1 mM nonessential amino acids. Confluent cultures were switched to mesodermal medium consisting of KO-DMEM supplemented with 20% (vol/vol) HyClone FBS, 1 mM t-glutamine, 0.1 mM β-mercaptoethanol, 0.1 mM nonessential amino acids, and 100 U/mL penicillin/100 μg/mL streptomycin [1% (vol/vol) stock solution] for 7 d with two media changes. Induced cultures were trypsinized, counted, and subcultured in mesodermal medium at 100,000 cells per cm² on tissue culture plates coated with 0.1% (wt/vol) gelatin in tissue culture water (passage 1). Upon reaching confluence, cells were trypsinized and subcultured at 10,000/cm² for up to 55 d (up to 10 or 11 passages).

Cell growth was documented by counting cell numbers at each passage, and cell morphology was documented by light microscopy (microscope IX81 mounted with Q-imaging camera; Olympus) by using MetaMorph imaging software (Molecular Devices).

Expression of surface antigens was analyzed at passages 1, 3, 5, 7, and 10. Cells were suspended at 10⁶ cells per mL, and 100 μL of the suspensions were incubated for 30 min on ice with antibodies Alexa Fluor 488 anti-human SSEA-1 (catalog no. 560172), Alexa Fluor 488 anti-human SSEA-4 (catalog no. 560308), Alexa Fluor 488 anti-human CD31 (catalog no. 558068), allopregocyanin anti-human CD34 (catalog no. 555824), fluorescein isothiocyanate anti-human CD44 (catalog no. 555478), phycoerythrin anti-human CD73 (catalog no. 550257), fluorescein isothiocyanate anti-human CD90 (catalog no. 555595), phycocerythrin anti-human CD166 (catalog no. 559263), and allopregocyanin anti-human CD271 (catalog no. 560326) obtained from BD Biosciences and Alexa Fluor 488 anti-human CD105 (catalog no. 323209) from BioLegend. Samples were washed and analyzed on the BD FACS Aria system using the BD FACS Diva software (BD Biosciences).

Expression was compared with unstained controls and designated positive (+) when 70% or more of the population expressed the specific marker; weakly positive (+/-) when 20–70% of the population expressed the specific marker; and negative (-) when less than 20% of the population expressed the specific marker.

Differentiation potential of H9- and H13-derived mesodermal progenitors was evaluated in monolayers and pellet cultures at passage 4. In parallel, control differentiation cultures were prepared from two lots of human bone marrow derived mesenchymal stem cells (BMSC; Lonza) that were expanded to passage 4 in BMSC expansion medium (high glucose DMEM supplemented with 10% (vol/vol) FBS, 1 ng/mL bFGF, 0.1 mM nonessential amino acids, and 1% (vol/vol) stock solution penicillin/streptomycin).

For monolayer cultures, cells were seeded into 24-well cell culture plates and cultured for 3 wk in (i) control medium (DMEM supplemented with 10% (vol/vol) HyClone FBS and 1% (vol/vol) Pen-Strep), (ii) osteogenic medium (control medium, supplemented with 1 μM dexamethasone, 10 mM β-glycerophosphate, 50 μM ascorbic acid-2-phosphate, (iii) adipogenic induction medium (DMEM supplemented with 10% (vol/vol) HyClone FBS, 1% (vol/vol) Pen-Strep, 1 μM dexamethasone, 10 μg/mL insulin, 200 μM indomethacin, 500 μM 3-isobuty1-1-methylxanthine; days 1–7 and 15–21 after reaching confluence) and adipogenic maintenance medium (DMEM supplemented with 10% (vol/vol) HyClone FBS, 1% (vol/vol) Pen-Strep, 10 μg/mL insulin; days 8–14 after reaching confluence). Osteogenesis was evaluated by histological staining of alkaline phosphatase activity (Fast Blue RR Salt; Sigma-Aldrich) and mineralization staining by von Kossa. Adipogenesis was assessed by Oil Red O staining of accumulated lipids.

Cell pellets were prepared by centrifuging 3 × 10⁶ cells 300 × g for 5 min and incubating 4 wk in control medium, osteogenic medium, and chondrogenic medium (DMEM supplemented with 1% (vol/vol) Pen-Strep, 100 nM dexamethasone, 50 μg/mL ascorbic acid-2-phosphate, 40 μg/mL t-proline, 1 × ITS, 1 mM sodium pyruvate, 10 ng/mL tumor growth factor β-3). For histological evaluations, pellets were washed in PBS and fixed in 3.7% (vol/vol) formaldehyde (24 h at room temperature), mounted in Histogel (Fisher Scientific), embedded in paraffin, and sectioned at 5 μm. Mineralization was evaluated by von Kossa stain, the presence of glycosaminoglycans (GAG) was evaluated by Alcian Blue stain, and the amounts of GAG and calcium were determined biochemically.

Results of staining were documented by light microscopy under the same illumination, capture time, and white balance settings for differentiation and control conditions in each cell line (microscope IX81 mounted with Q-Color 3 imaging camera, Olympus) by using MetaMorph imaging software (Molecular Devices). Collected images from all cell lines were converted to the same size and resolution by using Adobe Photoshop and combined into a single figure by using Adobe Illustrator software.

**Decellularized Bone Scaffolds.** Bone disks (4 mm diameter × 4 mm high) were prepared as described (1, 2). Briefly, trabecular bone was cored into 4-mm-diameter plugs from the subchondral region of carpometacarpal joints of 2-wk- to 4-mo-old cows (Green Village Packing). The plugs were washed with high velocity stream of water to remove the marrow from the pore spaces, followed by sequential washes in PBS, hypotonic buffer, detergent, and enzymatic solution to remove cellular material. At the end of the process, decellularized bone plugs were extensively rinsed in PBS, freeze-dried, and cut to 4 mm of length to obtain scaffolds for cell cultivation. The dry weights and exact scaffold density and porosity. Scaffolds in the density range of 0.38–0.44 mg/mm³ were sterilized in 70% ethanol and incubated in mesodermal medium overnight before cell seeding.

**Construct Assembly and Perfusion Bioreactor Culture.** H9 progenitors, H13 progenitors, and BMSC at passage 4 were suspended in culture medium at a density of 30 × 10⁶ cells per mL, and a 40-μL aliquot of the cell suspension was pipetted into the blot-dried...
scaffolds. Every 15 min, for 1 h, the scaffolds were flipped to facilitate uniform cell distribution. Each time, 5 μL of medium was added to prevent the cells from drying out. Seeded scaffolds were then placed in six well plates (1 scaffold per well) and incubated in 6 mL of osteogenic medium. The seeding efficiency, determined by evaluating the DNA content immediately after seeding, was between ~60% for hESC progenitors (determined in three separate trials) and ~80% for BMSC (similar to our previous studies). The initial cell density was determined before the transfer to bioreactors (day 3).

After 3 d, constructs were transferred to each of the perfusion bioreactors or six-well plates and cultured in osteogenic medium for up to 5 wk. Each bioreactor enabled uniform perfusion of six constructs, according to our established protocol (2). We selected a uniform flow rate of 3.6 mL/min corresponding to the interstitial velocity of 0.8 mm/s, which was set using a digital, low-flow, multichannel Ismatec peristaltic pump (Cole Palmer). These conditions were shown optimal in our previous work and close to settings used in other studies (2–4). Culture medium in the bioreactor was recirculated and maintained in equilibrium with the atmosphere in the incubator. These conditions established the oxygen concentration of 20% at the inlet of each construct. During culture, 50% of the medium volume was exchanged twice weekly with fresh medium. Medium samples were taken for biochemical assays at each medium change and stored at −80 °C. Cultured constructs were harvested after 3 and 5 wk of cultivation, cut in half, weighed, and processed for biochemical and histological analyses.

**Implantation Assay.** Safety and stability of H9-engineered bone constructs was assessed over 8 wk of s.c. implantation of cultured constructs (1 scaffold per group). After 7–8 wk (after the tumors formed by undifferentiated constructs reached a 5-mm diameter), mice were euthanized, and the tissues were harvested, washed, and fixed for μCT imaging and histological analyses.

**Biochemical Analyses.** For biochemical quantification of DNA and GAG content, samples were digested in 0.15 mL (pellets) or 1 mL (tissue constructs) of digestion buffer (10 mM Tris, 1 mM EDTA, and 0.1% (vol/vol) Triton X-100) containing 0.1 mg/mL proteinase K, overnight at 60 °C. Samples were repeatedly vortexed to facilitate digestion. Supernatants were collected and diluted as necessary to work in the linear range of the assays. DNA content was determined by using PicoGreen assay according to the manufacturer’s protocols (Invitrogen). GAG content was determined from aliquots of the digest by using the 1,9-dimethylmethane blue dye binding (DMMB) assay (5).

For determination of calcium content, pellets were extracted by incubation in 0.15 mL of 5% (vol/vol) trichloroacetic acid (Sigma) in water for 30 min with repeated pipetting, and the supernatants were analyzed by using Calcium (CPC) Liquicolor assay according to the manufacturer’s instructions (Stanbio Laboratory). For determination of alkaline phosphatase activity, tissue constructs were placed in 1 mL of lysis buffer [PBS, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 0.1 mg/mL phenylmethylsulfonfluoride, and 0.3% (vol/vol) aprotilin], maintained on ice, and disintegrated by steel balls in a Mini-bead beater (Biospec) over two 15-s cycles. The extracts were collected, centrifuged, and 50 μL of the supernatant was incubated in 50 μL of alkaline buffer and 50 μL of nitrophenyl-phosphate substrate solution, in microcentrifuge tubes at 37 °C until the development of yellow color. The reactions were stopped with 0.5 M NaOH, and the reaction times were noted. The absorbance at 405 nm was compared with a standard curve obtained from p-nitrophenol solutions of known concentrations to determine the alkaline phosphatase activity.

Aliquots of culture medium were evaluated for the concentration of osteopontin, using a human osteopontin ELISA kit developed according to the manufacturer’s instructions (R&D Systems).

**Histological Analyses.** Constructs were washed in PBS, cut in half, and fixed in 10% formalin for 1 d. One-half of each construct was processed for histological and immunohistochemical analysis by decalciifying for 2 d with Immunochemical solution (Decal Chemical), dehydration in graded ethanol solutions, paraffin embedding, sectioning to 5 μm, and mounting on glass slides (2–5 samples were prepared for each experimental group and time point). Samples were stained by using routine hematoxylin/eosin and Masson trichrome procedures.

For evaluation of bone matrix, immunohistochemical staining was performed by using primary antibodies (all purchased from Millipore) against osteopontin (rabbit polyclonal anti-osteopontin, catalog no. AB18700), bone sialoprotein (rabbit polyclonal anti-BSP II, catalog no. AB1854), osteocalcin (rabbit polyclonal anti-osteocalcin, catalog no. AB10911), and human nuclear antigen (mouse monoclonal anti-HNA, catalog no. MAB1281). Tissue sections were deparaffinized with Citrisolv (Fisher Scientific) and rehydrated with a graded series of ethanol washes. The antigens were retrieved by incubation in citrate buffer, according to the manufacturer’s protocols. The sections were then blocked with normal serum and stained with primary antibodies overnight, followed by the secondary antibody incubation, development with a biotin/avidin system, and counterstaining with hematoxylin. The serum, secondary antibody, and developing reagents were obtained from Vector Laboratories Vector Elite ABC kit (universal) (PK6200) and DAB/Ni Substrate (SK-4100). Negative controls were performed by omitting the primary antibody incubation step.

For hard-tissue histology, samples were fixed in 10% formalin (1–2 d) and dehydrated with sequential washes in ethanol (2 d at 70%, 2 d at 100% ethanol with twice daily solution changes) andtoluene (2 d with once daily solution change). Subsequently, the samples were washed in activated methylenecrylate (MMA) with daily changes of MMA solution for 4 d at 4 °C and then placed at 32 °C until the MMA was cured. Plastic-embedded sections were sectioned at 8 μm by using a Leica hard-tissue microtome. Ostex formation was evaluated by the traditional Goldner’s Masson trichrome staining.

Results of histological and immunohistochemical staining were documented by light microscopy under the same illumination, capture time, and white balance settings for all culture groups and negative staining controls (microscope BX53 mounted with DP72 camera; Olympus) by using DP2-BW imaging software (Olympus). Collected low-magnification images of hematoxylin/eosin, Masson trichrome, and immunohistochemical stains were processed in Adobe Photoshop to produce complete cross-section views of the tissues: Image levels were adjusted from 0–255 to 30–210 to enhance the contrast for viewing (except for osteocalcin and Masson trichrome), manually overlayed, and the top image brightness was adjusted (between 5 and 40) to minimize the differences in the border area. Images were merged, cropped to the same final canvas size for all groups, and combined into figures by using Adobe Illustrator.

High magnification images of Masson trichrome and Goldner trichrome stains (with no level adjustments), hematoxylin/eosin stains (with levels adjusted to 30–240), human nuclear stains (with levels adjusted to 30–255), and immunohistochemical stains (with levels adjusted to 30–210)
were cropped to the same final canvas size for all groups by using Adobe Photoshop and combined into figures by using Adobe Illustrator.

Semi-quantitative analyses of the histological and immunohistochemical sections were conducted by using ImageJ software (National Institutes of Health). For bone marker stains, each image was converted to an RGB Stack and the green stack was used. Thresholding was performed by using a value of 121 (selected based on the intensity of the negatively stained controls). Areas of the newly deposited tissue were manually selected, and the fractional area staining positive was measured for each sample in a complete cross-section. For osteoid stain, areas of positive (red) stain were manually selected, and the fractional area was measured for each sample.

**μCT imaging.** μCT was performed by using a modification of our developed protocol (6) on scaffolds before culture, in vitro cultured constructs, and explanted tissues. After fixation with glutaraldehyde, the samples were aligned along their axial direction and stabilized in a 15-mL centrifuge tube that was clamped within the specimen holder of a vivaCT 40 system (SCANCO Medical). The full 4-mm length of each construct was scanned at isotropic 21-μm resolution. The total bone volume (BV), consisting of the matrix in the scaffold and the new mineralized bone, was obtained by a global thresholding technique that selectively detects only the mineralized tissue. The bone volume fraction (BV/TV) was calculated by dividing the BV by the total volume of the sample. Spatial resolution of this full voxel model was considered sufficient for evaluating the microarchitecture of the bone tissue samples.

**Statistical Analyses.** Multiway analysis of variance (ANOVA) was used to compare different groups at the same time point and to compare different time points for any given group. Tukey’s post hoc analysis and the STATISTICA software were then applied to individual comparisons, with \( P < 0.05 \) being considered as significant.

**Fig. S1.** Characterization of the hESC-derived progenitors used in experiments. (A) Growth and morphology of hESC-derived progenitors. Adherent progenitors, derived from H9 and H13 lines, exhibited continuous growth for up to 10 passages (i) and exhibited fibroblastic morphology (ii, H9; iii, H13). (Magnification: 100×.) (B) Mesenchymal differentiation potential of hESC-progenitors and BMSC. H9- and H13-progenitor potential for osteogenesis was evidenced by positive staining of alkaline phosphatase activity (purple) in monolayer cultures and by matrix mineralization (von Kossa staining, black) in monolayer, and pellet cultures were stimulated with osteogenic medium (Insets, cultures in control medium). Mineralization was confirmed by biochemical evaluation of the pellet calcium content, which was significantly increased in osteogenic medium (Ost) compared with control (Ctrl) and chondrogenic (Chond) media. Osteogenesis of hESC-derived progenitors was comparable to that of BMSC. Weak chondrogenic potential was detected in H9-progenitor pellets compared with BMSC pellets by positive staining of glycosaminoglycans (Alcian Blue) and by determination of glycosaminoglycans content, which was significantly increased in chondrogenic medium. Similarly, weak adipogenic potential of hESC-progenitors compared with BMSC was observed by positive staining of lipid vacuoles (Oil Red O) in monolayer cultures stimulated with adipogenic medium. Biochemical data represent averages of 3–6 measurements ± SD (*P < 0.05; *, a statistically significant difference from other groups). (C) Surface antigen expression pattern of hESC progenitors. Expression at specific passage was designated positive (+) when 70% or more of the population expressed the specific marker; expression was designated weakly positive (+/−) when 20–70% of the population expressed the specific marker; expression was designated negative (−) when less than 20% of the population expressed the specific marker.
Fig. S2. Effect of bioreactor cultivation on tissue development from H13 progenitors. Both DNA content per ww (A) and AP activity (B) significantly increased in the bioreactor group from week 3 to week 5 of culture and were found to be significantly higher compared with the static group after 5 wk of culture. Cumulative osteopontin (OPN) release into culture medium was significantly higher during the first week of culture (medium change 1) and remained high compared with the static group throughout the culture (C). Data represent averages of 2–4 measurements ± SD (P < 0.05; *, a statistically significant difference between the groups; $, a statistically significant difference between week 3 and week 5). Positive effects of bioreactor culture were corroborated by histological analyses (D), showing denser tissue deposition in the bioreactor group and the presence of collagenous matrix (positive Masson Trichrome staining). Black T lines at the image edges mark the position where low magnification micrographs were overlayed.
Fig. S3. Expression of bone markers in tissue constructs engineered using H9 progenitors, H13 progenitors, and BMSC under static and perfusion conditions. (A) Low magnification images showing homogenous expression of bone markers in engineered tissue. Bioreactor-engineered tissue from H9-progenitors and BMSC stained strongly positive for bone markers osteopontin (Top), bone sialoprotein (Middle), and osteocalcin (Bottom). Insets represent negative staining controls. Black T lines at the image edges mark the position where micrographs were overlayed. Minimal staining was observed in statically cultured groups at weeks 3 and 5. Interestingly, after 3 wk of culture, less homogenous matrix deposition was noted in BMSC compared with H9-progenitor bioreactor cultures. (B) Expression of bone markers in engineered tissue from H13 progenitors. Bioreactor-engineered tissue from H9 progenitors stained strongly positive for bone markers osteopontin (Top), bone sialoprotein (Upper Middle) and osteocalcin (Lower Middle). Insets represent negative staining controls. Minimal staining was observed in statically cultured groups at weeks 3 and 5. New osteoid deposition (Bottom; red color) was noted in both groups after 3 wk, and in the bioreactor group after 5 wk of culture. (C) Low magnification images showing expression of bone markers in engineered tissue from H13 progenitors. Bioreactor-engineered tissue from H13 progenitors and H13 progenitors stained strongly positive for bone makers osteopontin (Top), bone sialoprotein (Middle), and osteocalcin (Bottom). Insets represent negative staining controls. Minimal staining was observed in statically cultured groups at weeks 3 and 5. New osteoid deposition (Bottom; red color) was noted in both groups after 3 wk, and in the bioreactor group after 5 wk of culture. (C) Low magnification images showing expression of bone markers in engineered tissue from H13 progenitors. Bioreactor-engineered tissue from H13 progenitors and H13 progenitors stained strongly positive for bone markers osteopontin (Top), bone sialoprotein (Middle), and osteocalcin (Bottom). Insets represent negative staining controls. Minimal staining was observed in statically cultured groups at weeks 3 and 5. New osteoid deposition (Bottom; red color) was noted in both groups after 3 wk, and in the bioreactor group after 5 wk of culture.
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**Fig. S4.** Engineered bone mineralization. Reconstructed 3D μCT images of the tissue engineered bone constructs from H13 progenitors before and after 5 wk of cultivation indicated formation of mineralized tissue (**A**). Bone structural parameters were determined by μCT analysis and indicated bone maturation during in vitro culture (**B**). Bone volume, bone volume fraction, and trabecular thickness increased significantly in both groups, in contrast to trabecular spacing, which decreased significantly in both groups, indicating bone maturation. Data represent averages ± SD (n = 3–4, P < 0.05; *, statistically significant differences from initial values within the same group).

**Fig. S5.** Close examination of engineered bone tissue after explantation. Scaffold seeded with H9 progenitors and H9-engineered bone exhibited loose connective tissue and denser bone-like tissue upon closer examination. Explanted samples were surrounded by loose connective tissue capsule. Black T lines at the image edges mark the position where low magnification micrographs were overlayed. At higher magnification (**Top Right**), the presence of functional microvessels containing red blood cells was evident in the edge and interior regions of the scaffold (marked by arrows). In addition, external regions of the scaffolds indicated the initiation of remodeling process, evidenced by invasion of multinuclear osteoclastic cells overlaying and degrading scaffold surfaces (marked by asterisks). Brackets denote representative positions of high-magnification images. Human origin of the cells was confirmed by positive staining of human nuclear antigen (Bottom) both in scaffolds seeded with H9 progenitors as well as H9-engineered bone (brown color).